Receptors during Neuromuscular Junction Formation in *Drosophila* Embryos

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Changes in the distribution and density of transmitter receptors in the postsynaptic cell are required steps for functional synapse formation. We raised antibodies against *Drosophila* glutamate receptors (DGluR-II) and visualized the distribution of receptors during neuromuscular junction formation in embryos. In wild-type embryos, embryonic development is complete within 22 hr after egg lying (AEL) and neuromuscular junction (NMJ) formation begins at 13 hr AEL. At the time of initial synapse formation, DGluR-IIs appeared as clusters closely associated with some muscle nuclei. Subsequently, these nonjunctional clusters dispersed while DGluR-IIs accumulated at the junctional region. In a paralytic temperature-sensitive mutant, *para^{ts1}*, neural activity decreases drastically at restrictive temperatures. When neural activity was blocked throughout synaptogenesis by rearing embryos at a restrictive temperature prior to the beginning of synaptogenesis, 12 hr AEL, the dispersal of extrajunctional clusters was significantly suppressed and no accumulation of receptors at the junction was observed at 22 hr AEL. However, when neural activity was blocked later, by rearing embryos at a restrictive temperature from 13 hr AEL, DGluR-IIs did not accumulate at the NMJ, although extrajunctional clusters dispersed normally. These findings suggest that the neural activity differentially regulates dissipation of receptor clusters in the nonjunctional region and accumulation of receptors at the junctional region.   © 1997 Academic Press

INTRODUCTION

During development of the vertebrate neuromuscular junction (NMJ) profound alterations in the distribution and density of acetylcholine receptors (AChRs) take place in the postsynaptic muscle cells (reviewed by Schuetze and Role, 1987; Hall and Sanes, 1993). Before neuronal contact, AChRs are distributed uniformly over the entire surface of the muscle cell, with occasional high density patches. After neuromuscular contact, AChRs rapidly accumulate at the postsynaptic membrane. The density of extrajunctional AChRs, however, decreases gradually sometime later by a process controlled by muscle activity (Lømo and Rosenthal, 1972). These changes underlie the formation of functional synapses and are key processes in synapse development.

To examine whether neuronal activity affects receptor distribution and density at the NMJ during development, we used a paralytic *Drosophila* mutant (*para*). The *para* gene encodes a functionally predominant class of sodium channels (Loughney *et al.,* 1989). A viable hypomorphic allele, *para^{ts1},* shows temperature-sensitive paralysis in larvae

and adults (Suzuki *et al.,* 1971) and synaptic transmission at the larval and embryonic NMJ is blocked at restrictive temperatures (Wu and Ganetzky, 1980; Broadie and Bate, 1993c). Therefore, we should be able to decrease or block neural/synaptic activities at any stage of development by shifting the rearing temperature of embryos from a permissive to a restrictive range.

In wild-type *Drosophila melanogaster* raised at 25°C, embryonic development is complete within 22 hr after egg laying (AEL) (the end of stage 17 by Campos-Ortega and Hartenstein, 1985). The neurotransmitter at this NMJ has been identified as L-glutamate (Jan and Jan, 1976). NMJ formation begins as soon as the motor nerve contacts the muscle surface at 13 hr AEL (stage 16). In longitudinal ventral muscles (muscle numbers 6 and 7), responses to focal iontophoretic application of L-glutamate are homogeneously distributed at the time of initial nerve contact and become restricted to the nerve contact region. No sensitivities are detected in the extrajunctional area after 15 hr AEL (Broadie and Bate, 1993a). Miniature endplate currents (mepcs) are first detectable at 16 hr AEL and the mean amplitude in-

creases progressively toward 22 hr AEL (Kidokoro and Nishikawa, 1994). These findings strongly suggest that postsynaptic receptors are accumulated at the NMJ during the period between 13 and 22 hr AEL and demonstrate that invertebrates share, with vertebrates, an apparently similar mechanism for distributional changes of receptors during synaptogenesis.

To visualize the distribution of receptors in the body-wall muscles of embryos, we raised antibodies against a *Drosophila* somatic musculature glutamate receptor subunit (DGluR-II, Schuster *et al.,* 1991). During embryonic development in wild-type, receptor redistribution occurred as documented in vertebrates *in vivo* (Dahm and Landmesser, 1991) and *in vitro* (Anderson and Cohen, 1977; Kuromi and Kidokoro, 1984). DGluR-IIs were found clustered in the non-nerve-contacted areas at the beginning of synaptogenesis. As synapse formation proceeded, these clusters dispersed and DGluR-IIs were found at the NMJ. By rearing *para^{ts1}* embryos, at restrictive temperatures, starting at different times during synaptogenesis, we have found that neural activity at a certain period of development profoundly affects the distribution of DGluR-IIs.

MATERIALS AND METHODS

Fly stocks. All fly stocks were raised at 25°C on standard *Drosophila* food. Canton-S was used as the wild-type. *para^{ts1}* was the gift of Dr. C.-F. Wu (University of Iowa, Iowa City).

Production and purification of antibodies. Two linear peptides, GSRRSSKEKSRSKTVS (residues 891–906, DM2) and YKSPPEPKNQF (residues 537–547, DS58), were synthesized. A cysteine residue was attached to the N-terminus of each peptide to facilitate coupling to keyhole limpet hemocyanin (Pierce, Rockford, IL) by *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce, Rockford, IL). The resultants of each conjugate were mixed with Freund's complete adjuvant and inoculated subcutaneously into rabbits (100 μg each). The second injection was carried out 2 weeks after the first and the third injection was given 10 days after the second. After the third subcutaneous injection, each antigenic peptide dissolved in 0.9% sodium chloride saline (100 μg/ml) was intravenously injected once a week for three times as described by Harlow and Lane (1988). Rabbits were bled 1 week after the last injection. Two series of antibodies against DM2 and DS58 were affinity-purified from the collected antiserum on Sepharose 4B columns (Pharmacia LKB, Piscataway, NJ) coupled with each peptide, as recommended by the manufacturer.

Immunoblotting. Immunoblotting was performed as described by Harlow and Lane (1988). Crude lysates were prepared from newly hatched larvae and staged embryos with lysis buffer as described by Gillespie and Wasserman (1994), electrophoresed on a 7.5% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking with Block Ace (Dainippon Seiyaku, Osaka, Japan), the membrane was treated with 0.5 μg/ml affinity-purified antibodies in a Block-Ace overnight at 4°C. To visualize the reacted band, biotinylated second antibodies were used in the ECL Western blot system (Amersham) as recommended by the manufacturer. For antibody preabsorption, the antibodies were incubated overnight at 4°C with 500 μg/ml of the respective antigenic peptide.

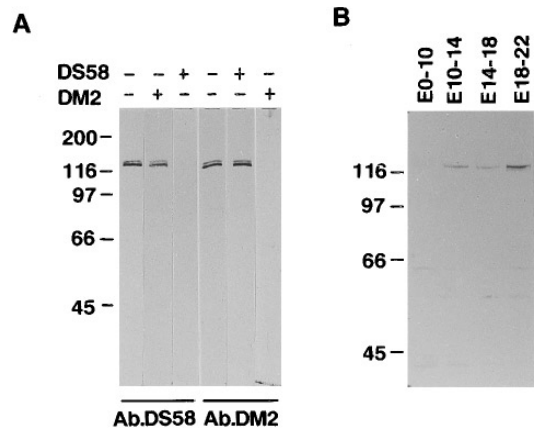*Immunofluorescence study.* Flies were allowed to lay eggs on

FIG. 1. (A) Characterization of affinity-purified antibodies directed to the C-terminal (Ab. DM2) and the N-terminal (Ab. DS58) regions of DGluR-II. (B) Expression of DGluR-II during embryonic development. Crude lysates 10 μg/lane from hatched first instar larvae (A) and 5 μg/lane from staged embryos (B) were subjected to immunoblotting analysis with affinity-purified antibodies. (A) DS58 and DM2 were the antigen peptides with which antibodies were preabsorbed on immunoblotting. Immunoreactive bands (123 kDa) were completely abolished by preabsorption of antibodies with their corresponding peptides. (B) DGluR-II expression was examined with Ab. DM2. Crude lysates were obtained from embryos at 0 to 10 hr AEL (E0–10), 10 to 14 hr AEL (E10–14), 14 to 18 hr AEL (E14–18), and 18 to 22 hr AEL (E18–22), respectively. Positions of the molecular weight (kDa) standards are indicated on the left.

the agar plate coated with dry yeast/grape juice for 30 min. Developmental times are indicated in hours after egg laying (AEL) and developmental stages were confirmed by morphological criteria (Campos-Ortega and Hartenstein, 1985). According to the previous study (Bate, 1990), we defined muscle and unfused myoblasts by position and by their morphology. Whole-embryo preparations were made as described by Kidokoro and Nishikawa (1994) and fixed in cold Bouin's fixative (75 ml of saturated picric acid, 25 ml of 37% formalin, and 5 ml of glacial acetic acid) on ice for 1–12 hr. Junctional DGluR-II immunoreactivities were clearly observed in 1 hr of fixation, while visualization of extrajunctional DGluR-II immunoreactive clusters required a longer fixation period (up to 12 hr). A longer fixation usually increased the background level and then illuminated nerve terminals, and dotted labelings were observed. After washing, the preparations were blocked in the Block Ace containing 0.2% Triton X-100 and then incubated overnight at 4°C in a mixture of primary antibodies, rabbit anti-DGluR-II (Ab. DM2 or Ab. DS58) (1 μg/ml), and goat anti-HRP IgG (Cappel, Durham, NC) (10 μg/ml). In *Drosophila,* the anti-HRP antibody binds to antigens exclusively located in neuronal membranes (Jan and Jan, 1982). The mixture of second antibodies consisted of rhodamine-conjugated donkey anti-goat IgG (Chemicon, Temecula, CA) (1:200), to visualize nerve terminals, and biotinylated donkey anti-rabbit IgG (Jackson, West Grove, PA) (1:1000). Then, preparations were incubated with fluorescein-conjugated streptavidin (Jackson) (1:1000) and the DNA dye DAPI (Molecular Probes, Eugene, OR) (0.1 μg/ml), for staining nuclei. All DGluR-II immunoreactivities were abolished when normal rabbit IgG (1 μg/ml) or preabsorbed anti-DGluR-II antibodies were used for incubation.
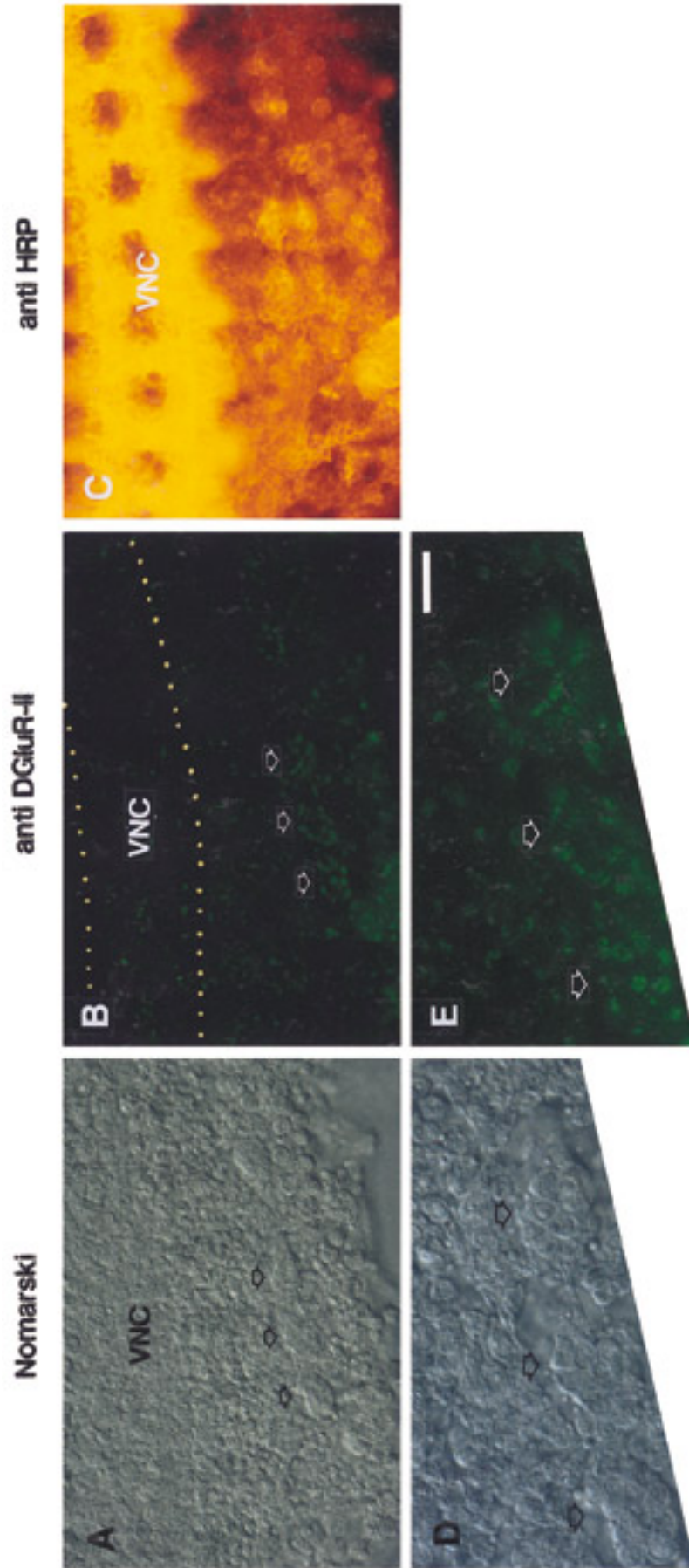
FIG. 2. Distribution of DGluR-II immunoreactivity in a dissected embryonic fillet at 10.5 hr AEL. Nomarski and corresponding immunofluorescent images. In Drosophila, anti-HRP antibody binds to antigens exclusively located in neuronal membranes (Jan and Jan, 1982). Dotted lines in (B) delineate the border of VNC. (D and E) Higher-magnification view of aggregated myoblasts and their DGluR-II immunoreactivities (large arrows) in (A) and (B). The scale bar shown in (E) corresponds to 25 μm (A–C) and 10 μm (D and E).

RESULTS

Characterization of Antibodies and Immunoblotting Study

Two types of ionotropic glutamate receptors in *Drosophila,* DGluR-I (Ultsch *et al.,* 1992), and DGluR-II (Schuster *et al.,* 1991), have been cloned and DGluR-II gene transcripts have been shown to be abundant in body-wall muscles. Two peptides corresponding to the putative extracellular region at the N-terminus (residues 537–547; DS58) and the putative intracellular region at the C-terminus (891–906; DM2) of the deduced amino acid sequence of a cloned DGluR-II cDNA were synthesized and used as antigens for raising polyclonal antibodies. Two series of polyclonal antibodies directed against each peptide (Ab. DS 58 and Ab. DM2) were affinity-purified on affinity columns conjugated with each peptide. The specificity of antibodies was assessed by an immunoblotting of crude lysate prepared from hatched larvae (Fig. 1A). On immunoblot analysis, both antibodies detected identical immunoreactive bands, with an estimated molecular weight of 123 kDa. The immunoreactive band disappeared when the antibody was preabsorbed with the corresponding peptide (500 μg/ml). These results show that the two antibodies react at a high degree of specificity with DGluR-II. The estimated molecular weights of 123 and 125 kDa are slightly larger than that predicted from the deduced amino acid sequence of the receptor (108 kDa; Schuster *et al.,* 1991). This difference may result from posttranslational modification, such as glycosylation, at the possible N-glycosylation sites of DGluR-II and the doublet band observed in Fig. 1A reflects variant modification of the protein. Since the antibody directed at the C-terminal region (Ab. DM2) always produced better staining in immunofluorescence studies, we used this antibody exclusively. Expression of DGluR-II during embryonic development is shown in Fig. 1B. DGluR-II immunoreactivity appeared during the period between 10 and 14 hr AEL. Although the expression level was unchanged until 18 hr AEL, it increased thereafter toward hatching.

Developmental Changes of Glutamate Receptor Distribution in Wild-Type Embryos

DGluR-II immunoreactivity in individual mesodermal cells before fusion. Since immunofluorescence was enhanced by longer fixation, preparations were fixed for 12 hr to show extrajunctional clusters, although it also increased background immunoreactivity. For junctional clusters, preparations were fixed for 1 hr to reduce the background. In the *Drosophila* embryo, axogenesis and initial outgrowth of pioneering axons from the VNC take place before formation of muscle fibers. At 9.5 hr AEL (Campos-Ortega and Hartenstein, 1985) somatopleura, which give rise to the somatic musculature, consist of a layer of unfused spheroidal mesodermal cells without organization within the segment. In a dissected embryonic fillet at 10.5 hr AEL, DGluR-II immunoreactivity was observed in a layer of unfused sphe-

roidal mesodermal cells and in longitudinal tracts of the ventral nerve-cord (VNC in Fig. 2). The DGluR-II immunoreactivity in the VNC became more intense at later stages (see Fig. 3B). As shown in Fig. 2D, some of the unfused spheroidal mesodermal cells aggregated at this stage (large arrows), and these aggregates seem to become muscle fibers at later stages. DGluR-II immunoreactivity was observed both in aggregated individual myoblasts and in surrounding cells (Fig. 2E). These results show that glutamate receptors already exist in myoblasts before fusion. Aggregated myoblasts fuse to become syncytial fibers by 11 hr AEL, and the complete muscle fiber pattern forms by 13 hr AEL (Johansen *et al.,* 1989). Currie *et al.* (1996) also reported that DGluR-II mRNA was first expressed sometime before 9.5 hr AEL in an *in situ* hybridization study.

As shown in Figs. 3A and 3D, developing muscle fibers (large arrows) were observed at 12.5 hr AEL, although a few unfused myoblasts were still present (asterisks). The growth cone of intersegmental nerves (ISN; arrows) arrived near the dorsal midline, and several branches of the segmental nerve (SN; double arrowheads) also appeared (Fig. 3C). At this stage, DGluR-II immunoreactivity in fused muscle fibers became more intense and formed clusters associated with some muscle nuclei (arrowheads in Fig. 3E). Moreover, DGluR-II immunoreactivity was found to be accumulated along longitudinal tracts of the VNC (Figs. 3B and 3E), suggesting the existence of DGluR-II-positive neurons or glia cells. DGluR-II immunoreactivity in the VNC disappeared by 17 hr AEL at latest (unpublished observation). The *in situ* hybridization study showed that DGluR-II transcripts were abundant in somatic muscles but not in the VNC (Schuster *et al.,* 1991; Currie *et al.,* 1996). This discrepancy cannot be resolved at this moment.

Dispersal of extrajunctional receptor clusters and receptor accumulation at the NMJ. By 13.5 hr AEL, the ISNs reach the dorsal midline and embryos show spontaneous movements (Johansen *et al.,* 1989; Broadie and Bate, 1993a). DGluR-II immunoreactivities were found to form clusters associated with some muscle nuclei at 14 hr AEL (Figs. 4A–4C). The clusters had an oval shape with the longer diameter being 3.6 ± 0.9 μm (mean ± SD; $n = 46$; Fig. 4G).

DGluR-II clusters became smaller but their numbers increased by 17 hr AEL (Figs. 4D and 4F), suggesting that large clusters dispersed into small aggregates. In fact, smaller DGluR-II clusters, 1.5 ± 1.0 μm ($n = 42$) in the longer diameter, were observed at 17 hr AEL (Fig. 4G). However, we could not exclude the possibility that small receptor clusters were newly formed rather than larger clusters being dispersed into small ones. In the negative control experiment using normal rabbit IgG instead of Ab.DM2, we did not observe such cluster-like (larger than 0.5 μm in length) immunoactivities but only a dotted (or spot-like) background (not shown).

During the period between 17 and 22 hr AEL, extrajunctional receptor clusters disappeared and DGluR-IIs accumulated at the synaptic region without clear changes in the presynaptic structure. In the immunofluorescence staining, distinct junctional DGluR-II clusters appeared and most of
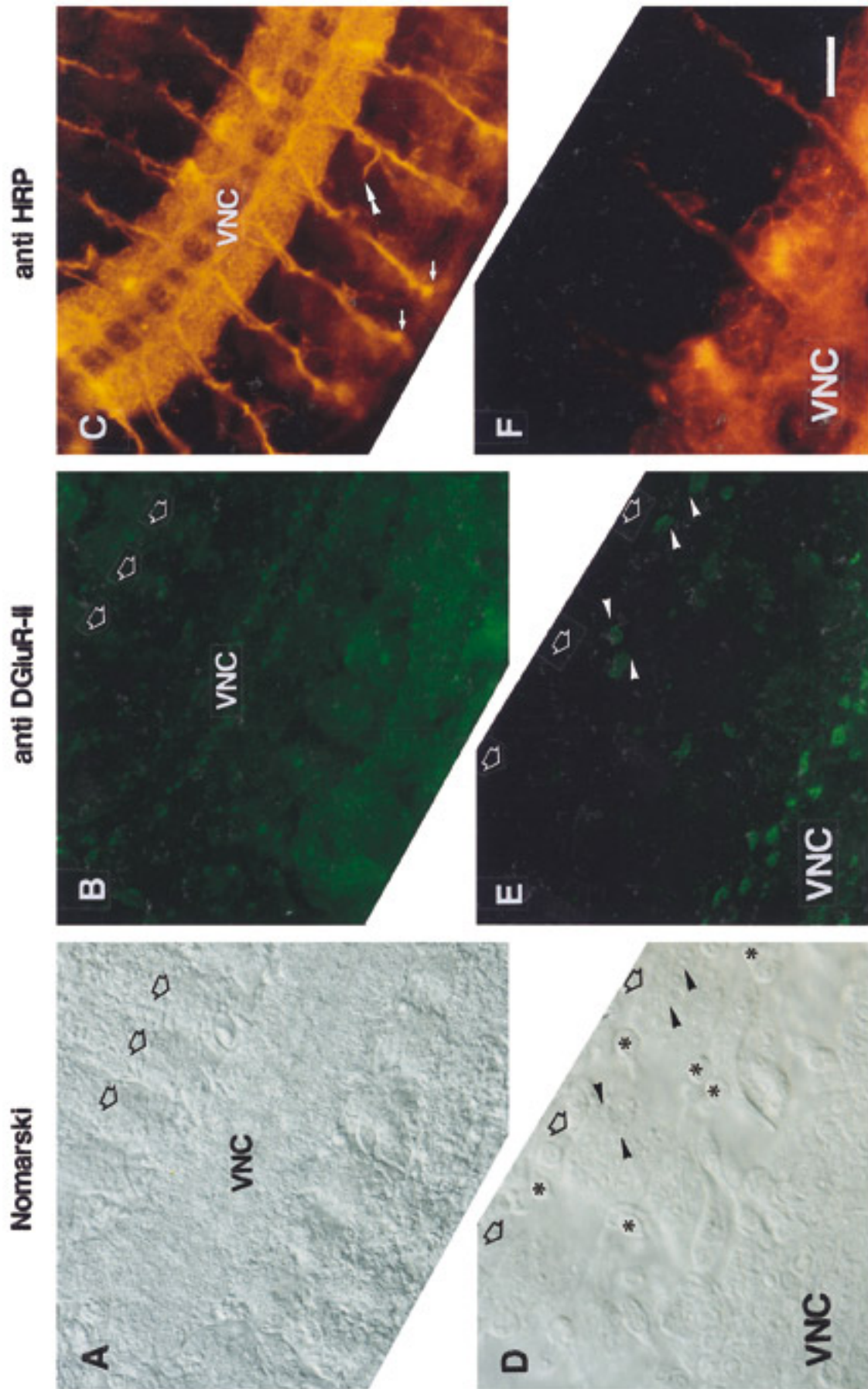
FIG. 3. Distribution of DGluR-II immunoreactivity in a dissected embryonic fillet at 12.5 hr AEL. Nomarski and corresponding immunofluorescence images. Each broad arrow in (D) and (E) points to corresponding muscle fibers indicated by broad arrows in (A) and (B). Arrows and double arrowhead in (C) indicate the growth cone of the ISN and the branch of the SN, respectively. (D–F) Higher-magnification views from (A–C). DGluR-II immunoreactivity forms clusters and associates with some nuclei (arrowheads) of ventral longitudinal muscle fibers (large arrows). Asterisks indicate unfused myoblasts. The scale bar in (F) corresponds to 25 μm (A–C) and 10 μm (D–F).
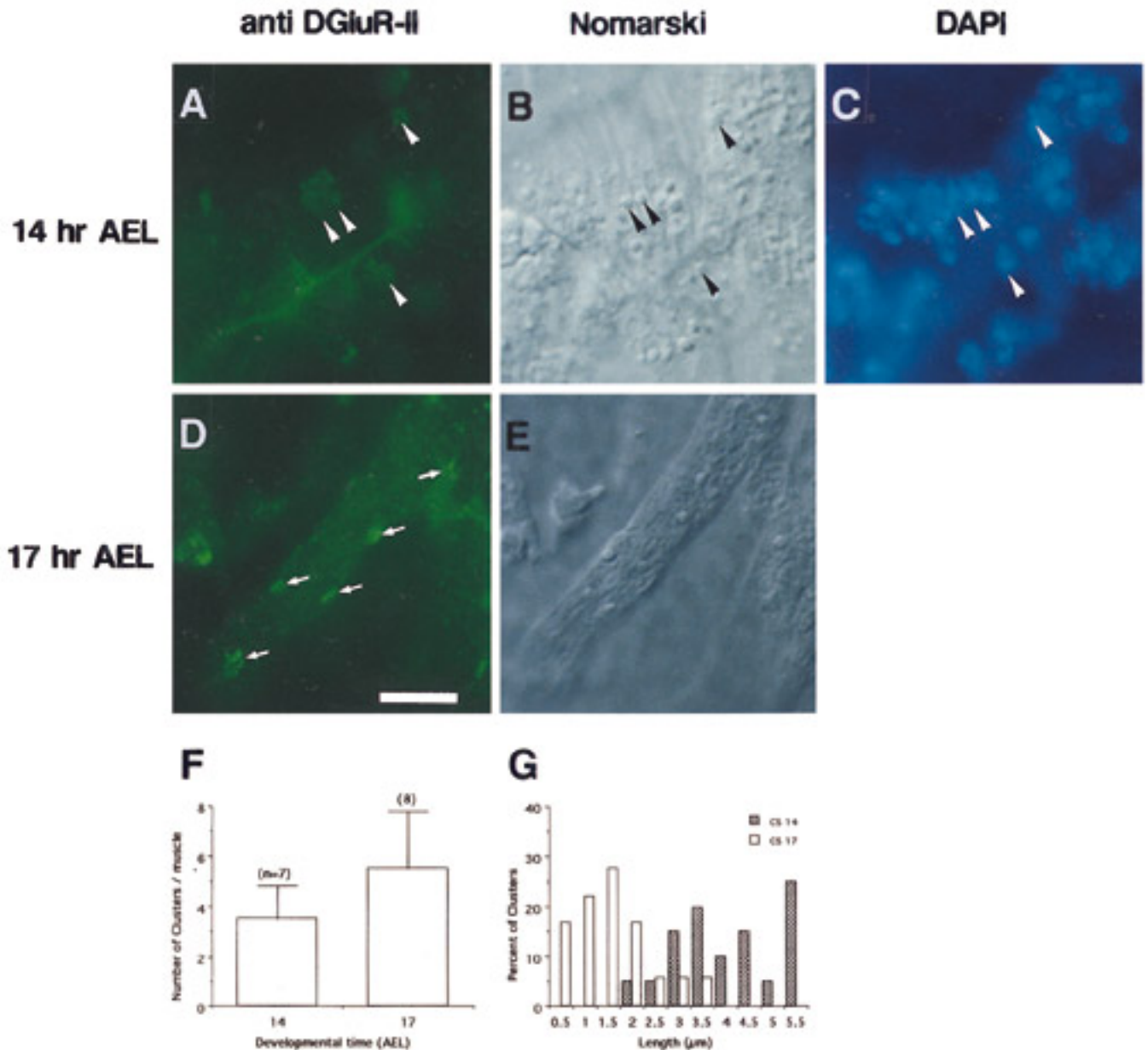
FIG. 4. Extrajunctional clusters of DGluR-II immunoreactivities in muscle fibers at 14 and 17 hr AEL. (A–E) Immunofluorescence and corresponding Nomarski images of dorsal muscle fibers in an embryo at 14 (A, B, and C) and 17 hr AEL (D and E). DGluR-II immunoreactivity formed large clusters near some of muscle nuclei (arrowheads) at 14 hr AEL. (G) Percent histogram of the length of DGluR-II clusters. Number and length of clusters were counted from muscles 6, 7, 15, 16, and 17 (ventral) and muscles 1 and 2 (dorsal; see Johansen *et al.,* 1989 for muscle numbers). The scale bar in (D) corresponds to 10 μm (A–E). Each bar in (F) represents the mean $\pm$ SD. DAPI, the DNA dye for staining nuclei.

the extrajunctional clusters disappeared by 20 hr AEL. Figure 5 shows the distribution pattern of DGluR-II at 22 hr AEL. The distribution of DGluR-II immunoreactivities matched closely the shape of presynaptic terminals, and no staining was found, at this time, in extrajunctional regions

(Figs. 5B and 5E). We think that extrajunctional DGluR-II clusters disappear due to dissipation into the homogeneous distribution, and not due to suppression of receptor expression, because first, the total amount of DGluR-II protein did not decrease during the decay phase of extrajunctional
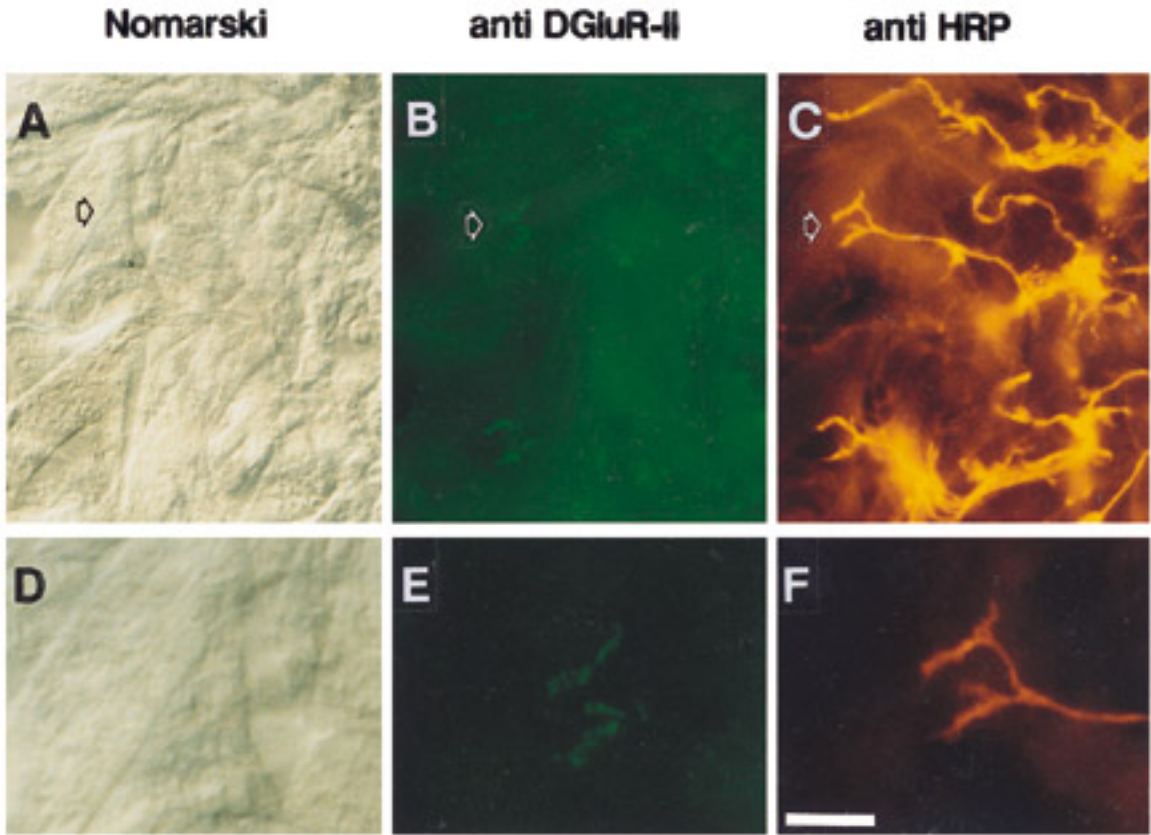
Nomarski   anti DGluR-II   anti HRP

FIG. 5. DGluR-II immunoreactivity accumulated at the NMJ by 22 hr AEL. Nomarski and corresponding immunofluorescence images. (D–F) Higher-magnification views of the junctional region on dorsal muscles indicated by large arrows (A–C). DGluR-II immunoreactivities correspond exactly to the terminal arborization. Preparations were fixed for 1 hr to reveal junctional clusters. Dotted labeling in the extrajunctional region was the immunofluorescence background but not clusters, judged from their length (see text). The scale bar in (F) corresponds to 25 μm (A–C) and 10 μm (D–F).

DGluR-II clusters (Fig. 1B; E14–18), and second, it has been reported that functional glutamate receptor channels exist abundantly in extrajunctional areas in late stage embryos and even in first instar larvae (Nishikawa and Kidokoro, 1995). It should be noted that we could not detect low and homogeneously distributed DGluR-IIs on muscle fibers using the present immunofluorescence technique.

Effects of Neural Activities on Distribution of DGluR-IIs during NMJ Formation

Extrajunctional clusters of DGluR-IIs remain when para^ts1 embryos are reared at restrictive temperatures. Mutations in the *para* locus decrease the level of neural activity and consequently reduced synaptic transmission at the NMJ (Wu and Ganetzky, 1980). The viable hypomorphic *para^ts1* allele decreases or blocks synaptic transmission at the embryonic as well as larval NMJ in a temperature-sensitive manner (Broadie and Bate, 1993c). In *para^ts1* embryos,

synaptic transmission occurs at 25°C, but is reduced at 30°C and completely blocked at 34°C (Broadie and Bate, 1993c).

para^ts1 embryos were shifted to the restrictive temperature of 32°C at 12 hr AEL (before nerve–muscle contact) and kept at this temperature for the remainder of embryogenesis. These embryos did not move spontaneously nor were their tracheae inflated. But, they showed normal muscle pattern formation and shortening of the VNC. When the immunoreactivity of DGluR-IIs was examined at 22 hr AEL by fixing embryos for 12 hr, receptor clusters were found at the extrajunctional region, as shown in Fig. 6A. Although we sometimes observed junctional clusters with low fluorescence intensities, we did not defined them as positive signals since clear receptor accumulation was not found at the NMJ when the embryos were fixed for 1 hr (not shown). In contrast, the wild-type embryos, reared at 32°C for 10 hr starting at 12 hr AEL, did not have receptor clusters in the extrajunctional area, and receptors were found clustered at the synaptic site when the immunoreactivity of DGluR-IIs was examined at 22 hr AEL by fixing

embryos for 1 hr (Figs. 6C and 6D). These wild-type embryos moved spontaneously, and their tracheae were inflated although they showed a reduction in hatching efficiency. When *para^{ts1}* embryos were reared at 30°C, a few clusters in the extrajunctional region remained, and receptors were localized at the synaptic site less frequently. These *para^{ts1}* embryos showed lower spontaneous movements and hardly hatched. When *para^{ts1}* embryos were reared at 25°C, clusters in the extrajunctional area disappeared, and receptors were localized at the synaptic site. These *para^{ts1}* embryos developed and hatched normally (Table 1). However, when embryos were reared at 34°C for 10 hr starting at 12 hr AEL, receptor clusters remained in the extrajunctional region, and receptor accumulation at the junction was observed neither in wild-type nor in *para^{ts1}* embryos (Fig. 6E). Neural activity is also reduced in wild-type at 34°C (Broadie and Bate, 1993c). We presumed that reduced neural activity prior to nerve–muscle contact (12 hr AEL) severely hindered neuromuscular junction formation. We should mention that such effects on extrajunctional clusters in wild-type embryos were not observed when they were shifted to 34°C after nerve–muscle contact (13 hr AEL).

Under these conditions, the number of receptor clusters progressively increased at higher temperatures in *para^{ts1}* embryos (Fig. 6E). The cluster size was small and not different between 30 and 32°C. However, at 34°C the average size of clusters was significantly larger ($P < 0.001$; Student's t test) than at 30 and 32°C (Fig. 6F and Table 2) and similar to that observed at 17 hr AEL in wild-type embryos reared at 25°C (see Fig. 4F).

Neural activity is required for receptor accumulation. As shown in Fig. 7A, in *para^{ts1}* embryos which were shifted to 34°C at 13 hr AEL, no accumulation of DGluR-IIs at the NMJ was observed. Whereas extrajunctional clusters were examined by fixing for 12 hr, and none of these embryos showed extrajunctional clusters (not shown). In these *para^{ts1}* embryos, spontaneous muscle contractions and peristaltic movements were observed up to 15 hr AEL (initial movements). However, during the period between 16 and 22 hr AEL, embryos barely moved, although they secreted cuticle and their tracheae were inflated. In wild-type embryos reared at 34°C, accumulation of DGlu-RIIs was observed at 22 hr AEL (Fig. 7C). As we mentioned above, neural activity is reduced in wild-type at 34°C, and junctional receptor accumulation was less frequent than in those reared at 25 and 32°C (see Figs. 5E and 6C). These wild-type embryos showed spontaneous movement throughout development after nerve–muscle contact (13 hr AEL; initial and late movements), they secreted cuticle, and their tracheae were inflated. But embryos hatched less frequently. When *para^{ts1}* embryos were reared at 25°C, DGluR-IIs formed junctional clusters like wild-type embryos (Fig. 7E). Since the *para^{ts1}* embryos reared at 34°C showed initial movements and their extrajunctional DGluR-II clusters disappeared, muscle activity during the period of initial movements may be related to the disappearance of extrajunctional clusters. On the other hand, subsequent neural activity during the period between 15 and 22 hr AEL was required

for accumulating DGluR-II at the NMJ (Table 1) and for late movements.

DISCUSSION

Extrajunctional DGluR-II Clusters in the Developing Muscles

By iontophoretic mapping, Broadie and Bate (1993a) reported previously that glutamate receptors were homogeneously distributed over the entire surface of muscle at the beginning of NMJ formation and disappeared by 15 hr AEL in the extrajunctional region. However, by visualizing glutamate receptors, we found that DGluR-II appeared as clusters at first in the extrajunctional region, and the clusters were observed up to 17 hr AEL. During *Drosophila* embryogenesis, individual myoblasts begin to aggregate by 10.5 hr AEL and fused to form syncytial muscle fibers by 11 hr AEL (Johansen *et al.,* 1989). At 13.5 hr AEL, glutamate receptor channel activities were detected by the cell-attached patch clamp technique (Nishikawa and Kidokoro, 1995). Similarly, iontophoretic application of glutamate produced inward currents at 13 hr AEL (Broadie and Bate, 1993a). Broadie and Bate (1993b) showed that initial receptor expression occurred independently of innervation. Our immunofluorescence data show that DGluR-II reactivity already exists at the myoblast stage (10.5 hr AEL). In accord with our findings, Currie *et al.* (1995) have reported that DGluR-II mRNA is first expressed by 9.5 hr AEL.

Concerning extrajunctional clusters, changes in the distribution of DGluR-IIs during NMJ formation in *Drosophila* embryos are similar to that observed in *Xenopus in vitro.* In embryonic *Xenopus* muscle cultures, AChRs form clusters in the muscle cell membrane regardless of the site of nerve contact (Anderson and Cohen, 1977). After nerve–muscle contact, extrajunctional clusters disperse into small speckles and small clusters emerge along the course of nerve contact (Kuromi and Kidokoro, 1984). In a similar way, DGluR-II immunoreactivities appear as clusters near some of nuclei of fused muscle fibers. At later stages, a larger number of smaller clusters appear without any relation to the position of nuclei. We presumed that the clusters finally distributed homogeneously in the extrajunctional region. Our presumption is based on the observation by the cell-attached patch clamp study in which extrajunctional glutamate receptor channel events are detected even in hatched larvae (Nishikawa and Kidokoro, 1995). As suggested previously, desensitization may be prominent in the extrajunctional receptors and less so in the junctional receptors. Consequently, iontophoretic mapping did not detect existing extrajunctional receptors. Although *in vivo* AChRs were thought to be homogeneously distributed in the muscle cells before nerve–muscle contact (Jacobson, 1991; Hall and Sanes, 1993), several lines of evidence indicate that AChR clusters are induced to form before nerve–muscle contact in chick muscles *in vivo* (Godfrey *et al.,* 1988; Fallon and Gelfman, 1989; Dahm and Landmesser, 1991). Incoming
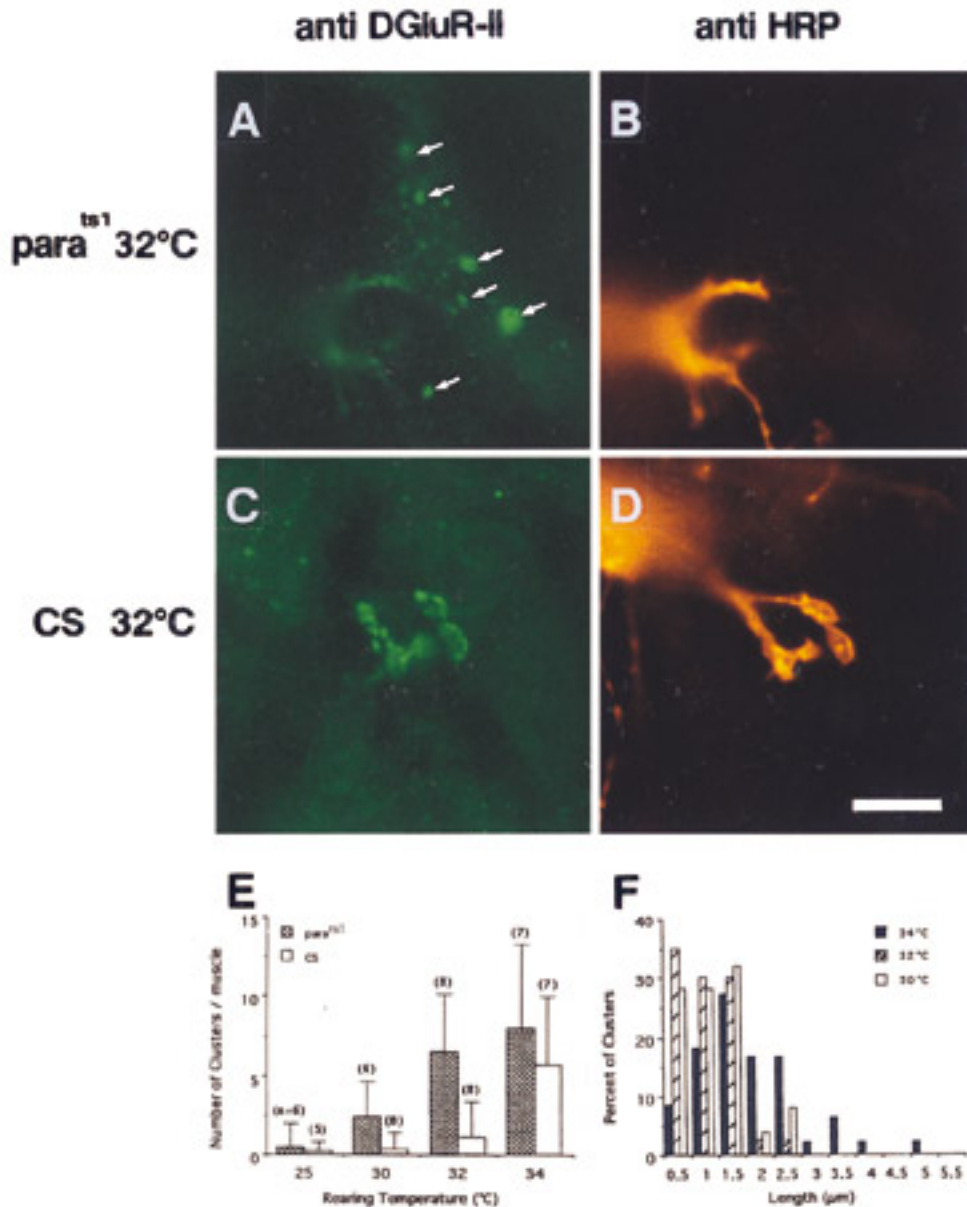
FIG. 6. Extrajunctional DGluR-II clusters did not disappear under conditions that suppressed neural activity from 12 hr AEL. (A–D) Immunofluorescence images of dorsal muscle at 22 hr AEL. (A and B) A *para*[ts1] embryo reared at 32°C for 10 hr from 12 hr AEL. Preparations were fixed for 12 hr to reveal extrajunctional clusters. (C and D) A wild-type embryo reared at 32°C for 10 hr from 12 hr AEL. Preparations were fixed for 1 hr to reveal junctional immunoreactivities. (E) The number of clusters per muscle in *para*[ts1] and wild-type embryos reared at various temperature. (F) The percent histogram of the length of clusters in *para*[ts1] embryos. Numbers and lengths of clusters were counted at 22 hr AEL as described in Fig. 4. The scale bar in (D) corresponds to 10 μm (A–D). Each bar in (E) represents the mean ± SD.

nerves seem to induce AChR clustering by releasing diffusible substance before contacting myotubes (Dahm and Landmesser, 1991). The mechanism responsible for inducing initial DGluR-II clusters in *Drosophila* muscles seems to be different from that in chick embryonic muscles since DGluR-II clusters appear without relation to the nerve terminal but are associated with some of muscle nuclei. The

initial appearance of DGluR-II clusters in *Drosophila* muscle fibers is comparable to the appearance of AChR clusters in cultured uninnervated myotubes or in denervated adult muscle of chicken where clusters appear near muscle nuclei (Frank and Fischbach, 1979; Ishikawa *et al.,* 1988). AChR clusters were seen as peaks of sensitivities, hot spots, in response to iontophoretically applied ACh in cultured unin-

TABLE 1

Effects of Neural Activity on Distribution
of Glutamate Receptors

Temperature shift at	Clusters		Movements	
	Extrajunctional	Junctional	Initial	Late
12 hr AEL				
para^ts1				
25°C	0/5	6/6	5/5	6/6
30°C	5/6	2/6	4/9	3/6
32°C	8/8	0/8	0/9	0/8
34°C	7/7	0/7	0/8	0/7
CS				
32°C	0/8	8/8	7/8	8/8
34°C	6/7	1/7	2/9	1/7
13 hr AEL				
para^ts1				
25°C	0/5	5/5	4/4	5/5
32°C	0/8	5/8	5/5	8/8
34°C	0/8	1/8	6/6	2/7
CS				
34°C	0/5	5/5	5/5	5/5

Note. Existence of clusters was examined in dorsal muscles 1, 2, 9, and 10. Number of (observed/examined) preparations is indicated.

nervated chick myotubes (Frank and Fischbach, 1979). Although we found extrajunctional receptor clusters by immunofluorescence, it is possible that these receptors are not functional. However, a mapping study of glutamate receptors by laser spot photostimulation with caged L-glutamate demonstrated that those clustered receptors are functional (Saitoe and Koshimoto, unpublished observation).

Concerning junctional clusters, iontophoretic mapping studies indicated that junctional clusters began to form from 15 hr AEL (Broadie and Bate, 1993a). However, we could not detect distinct clusters until 20 hr AEL. Our previous study with a whole-cell clamp technique presented results similar to those with iontophoretic mapping (Kidokoro and Nishikawa, 1994). Since electrophysiological detection of junctional receptors is more sensitive than the present immunofluorescence technique, we could not detect the early phase of junctional accumulation.

Neural Activity Is Required for Elimination of Extrajunctional DGluR-II Clusters and Accumulation of DGluR-IIs at the NMJ

para is a structural gene for a *Drosophila* sodium channel (Loughney *et al.,* 1989). Thus, it is possible to reduce, or block, neural activities in *para^ts1* mutant embryos in a temperature-dependent manner (Broadie and Bate, 1993c). A previous electrophysiological study showed that neural activity leads junctional accumulation of glutamate receptors (Broadie and Bate, 1993c). In the present study, we newly found that neural activity also regulates dissi-

pation of extrajunctional receptor clusters in addition to junctional receptor accumulation, probably by a different mechanism. We shifted *para^ts1* embryos from permissive to restrictive temperatures at 12 or 13 hr AEL for the remainder of embryogenesis. A *para^ts1* embryo shifted to restrictive temperatures at 13 hr AEL showed initial movements, spontaneous muscle contraction, and peristaltic movements up to 15 hr AEL, while a *para^ts1* embryo shifted to restrictive temperatures (especially to 32 or 34°C) at 12 hr AEL hardly showed initial movements and kept quiescent throughout development. By the end of embryogenesis (22 hr AEL), in *para^ts1* embryos which showed initial movements, extrajunctional clusters disappeared, while in *para^ts1* embryos which failed to show initial movements, extrajunctional clusters remained. These findings suggest that electrical activity of muscles might be causally related to dissipation of extrajunctional clusters. Since neural activity is also reduced in wild-type at 34°C (Broadie and Bate, 1993c), extrajunctional receptor clusters remained in the wild-type as well as in the *para* embryos when embryos were reared at 34°C from 12 hr AEL. Previously, Broadie and Bate (1993c) reported that when *para^ts1* embryos were reared at 34°C for 8 hr from 13 hr AEL, extrajunctional glutamate sensitivities remained homogeneously distributed over the muscle surface and were not localized at the NMJ. Their findings are consistent with our present results that glutamate receptors accumulate at NMJ in a neural activity-dependent manner. However, we did not find any extrajunctional receptor clusters when *para^ts1* embryos were reared at 34°C from 13 hr AEL.

We think that most of initial movements were neurally induced via synaptic transmission for the following reasons: first, the growth cone of motor axon contains glutamate at the time of nerve–muscle contact (Johansen *et al.,* 1989; Broadie and Bate, 1993a). At similar developmental stages, the growth cone releases ACh prior to contact with muscle cells in *Xenopus* (Young and Poo, 1983) and in chick (Hume *et al.,* 1983) embryonic cell cultures, and functional transmission starts as soon as the growth cone contacts muscle (Kidokoro and Yeh, 1982). Second, receptor channel currents were detected at 13.5 hr AEL by the cell-attached patch clamp technique (Nishikawa and Kidokoro, 1995),

TABLE 2

Effects of Neural Activity on Remained Cluster Size
in *para^ts1* Embryos

Temperature (°C)	Cluster size (μm)	n
30	0.8 ± 0.6	36
32	0.8 ± 0.5	41
34	1.5 ± 0.9	38

Note. para^ts1 embryos were shifted to restrictive temperatures at 12 hr AEL. The longer diameter of remained clusters is indicated by mean ± SD.
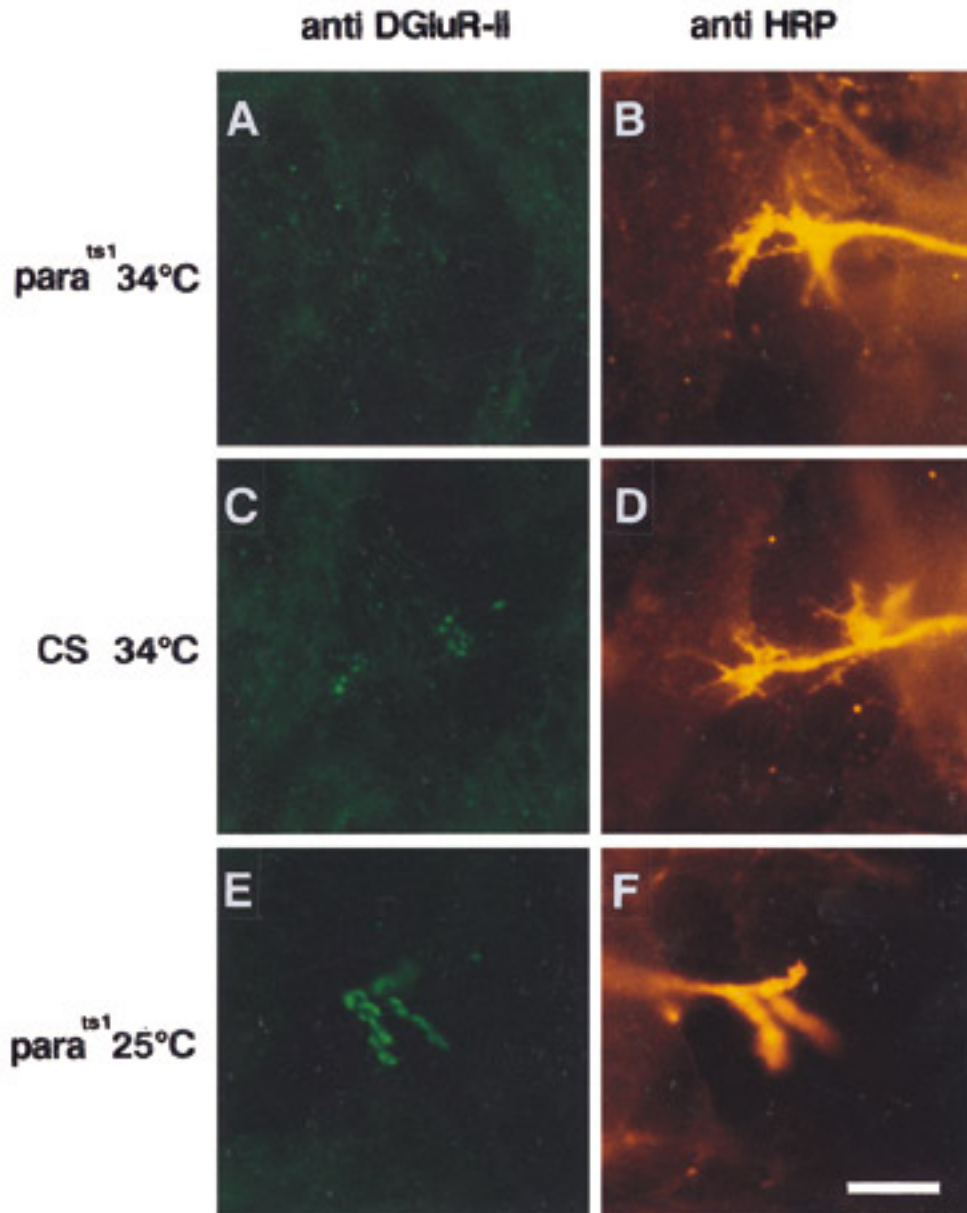
anti DGluR-II anti HRP

para^{ts1} 34°C

CS 34°C

para^{ts1} 25°C

FIG. 7. Junctional DGluR-II clusters did not accumulate under conditions that blocked neural activity from 13 hr AEL in *para^{ts1}* immunofluorescence images of anti DGluR-IIs (left column) and anti-HRP (right column) immunoreactivities at 22 hr AEL. All preparations shown here were fixed for 1 hr. (A and B) A *para^{ts1}* embryo reared at 34°C for 9 hr from 13 hr AEL. DGluR-II immunoreactivity was not observed at the junctional sites. (C and D) A control wild-type embryo. (E and F) A *para^{ts1}* embryo reared at 25°C. The scale bar in (A) corresponds to 10 μm (A–F).

and sensitivity to iontophoretically applied L-glutamate was detected in the extrajunctional region at this time (Broadie and Bate, 1993a). Third, in aneural *pros* mutants, in which peripheral motor nerves arrive at the muscle surface with a short delay, endogenous muscle contractions were observed infrequently during the period between 13 and 16 hr AEL (Broadie and Bate, 1993b). In dissected embryonic fillets of wild-type, argiotoxin, which blocks synaptic transmission

(Broadie and Bate, 1993a), prevented most endogenous muscle contractions and all peristaltic locomotory movements during the period between 13 and 16 hr AEL (unpublished observation). Therefore, by comparing the results from *para^{ts1}* embryos shifted to restrictive temperatures at 13 hr AEL with those at 12 hr AEL, we conclude that initial movements, which resulted from neural activity, contribute to dispersal of extrajunctional clusters of DGluR-II, and

accumulation of DGluR-IIs might require neural activity after 15 hr AEL.

The mechanism which dissipates receptor clusters in an activity-dependent manner is not yet known. In primary cultures of embryonic chick myotubes, electrical activity represses AChR biosynthesis (for review see Salpeter and Loring, 1985; Laufer and Changeux, 1989). However, if this repression of biosynthesis is also responsible for the dissipation of extrajunctional clusters in *Drosophila* embryos, the lifetime of DGluR-IIs must be short (a few hours) while the half-degradation time of nonsynaptic chick AChRs is longer (17–19 hr; Fambrough, 1979). Thus, it is not likely that a repression of receptor biosynthesis is involved in receptor dissipation in *Drosophila* embryos.

It has been proposed that junctional AChR clusters are formed at least partly by the trapping of freely diffusing AChRs (Anderson and Cohen, 1977; Kuromi and Kidokoro, 1984; Kidokoro and Brass, 1985). Recent experiments have shown that agrin, a basal lamina-associated protein, is responsible for inducing AChR clusters at the NMJ *in vivo* (Gautam *et al.,* 1996) as well as in cultured myotubes (McMahan, 1990). Alternative molecules that might be released from motor neurons and which may induce accumulation of DGluR-IIs at NMJ are CGRP (calcitonin gene-related peptide; Matteoli *et al.,* 1988; Hall and Sanes, 1993)- and neuregulin (Jo *et al.,* 1995)-like molecules. Both CGRP and neuregulin are synthesized by motor neurons and lead to an increase in AChR mRNA and protein levels. In particular, CGRP was shown to be released on stimulation (Uchida *et al.,* 1990). However, CGRP may not accumulate at the NMJ until some time after AChR cluster formation (for review see Hall and Sane, 1993). Although it is not clear whether the junctional clusters in *Drosophila* embryos contain newly synthesized DGluR-IIs, neuregulin- or CGRP-like molecules are attractive candidates which may regulate accumulation of DGluR-II at NMJ in a neural activity-dependent manner. We do not know about the molecules which regulate the accumulation of DGluR-IIs at the NMJ, but we speculate that neural activity directly controls production or transport or release of the molecule.

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